Enzyme-catalyzed Synthesis and Degradation of Biopolymers

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Abstract: The synthesis and degradation of biopolymers are cardinal reactions that take place to maintain homeostasis in biosystems. Fundamental biological reactions can be reproduced *in vitro* by means of enzyme-mediated processes. Recently, bioprocesses have been extensively adopted to synthesize representative biopolymers, including aliphatic polyesters, e.g., polyhydroxyalkanoate (PHA). In particular, the protein engineering of enzymes involved in PHA synthesis has made a great impact on the tailor-made synthesis of polymer materials with high performance. Biopolymers can also be synthesized *in vitro* by enzyme-catalyzed polymerization using synthetic enzymes and depolymerase-mediated reverse reactions. We will describe these advanced topics from the viewpoint of the boundary field between chemistry and biotechnology.

Keywords: Biopolymer, polyhydroxyalkanoate (PHA), PHA synthase, enzyme evolution, PHB depolymerase, lipase.

INTRODUCTION

Fossil resources, such as coal, petroleum, and natural gas, are not only consumed for energy and heat production, but also used as basic materials for the development of industrial systems. However, the increased consumption of fossil resources has led to global warming that is characterized by a marked increase of atmospheric CO_2 level as well as depletion of the resources. The last half century has witnessed the development of synthetic polymers from fossil resources, and 150 million tons of synthetic polymers are annually produced from fossil resources at present. However, although polymers have excellent functions for practical application, their high stability has led to an increase in the amount of polymer waste and consequently environmental pollution. Therefore, the development of environmentally sound alternatives to conventional polymers synthesized from fossil fuels is urgently desired to sustain the environment [1-5].

Biopolymers are eco-friendly materials because they are produced from renewable carbon sources via biological and/or chemical processes and after their use, they are biologically degraded and returned to the natural environment as renewable resources, such as CO_2 and biomass (Fig. **1**). Academic and industrial scientists have accelerated their efforts to develop biopolymers with the intent of solving problems associated with the use of petrochemical-based synthetic polymers. Biopolymers can be roughly divided into the following categories: (1) microbially synthesized polymers, such as polyhydroxyalkanoates (PHAs), polylysine, and polyglutamate; (2) polymers originating from animals and plants, such as polysaccharides and polyisoprenoids; and (3) chemically synthesized polymers, such as polylactide and polyaspartate. Among these biopolymers, PHAs, which are water-insoluble polyesters, are one of the most promising alternatives to conventional polymers [6-10]. PHAs show thermoplasticity in addition to biodegradability and biocompatibility. The fact that the physical and mechanical properties of PHAs can be regulated by varying monomer composition and some PHAs have properties comparable to petrochemical-based thermoplastics has resulted in various applications in industry, medicine, pharmacy, agriculture, and electronics [11].

In this review, we present enzyme-mediated bioprocesses for the synthesis of representative biopolymers in various polymerization systems by converting biological process (*in vivo*) into man-made process (*in vitro*). We also focus on the enzyme-catalyzed degradation of biopolyesters by their depolymerases.

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PROCESS CONVERSION OF BIOPOLYESTER: FROM WHOLE-CELL-CATALYZED SYNTHESIS TO CHEMOENZYMATIC SYNTHESIS

PHAs can be synthesized from renewable resources by *in vivo* biological processes. In 1925, Lemoigne discovered PHA in the form of poly(3-hydroxybutyrate) (PHB) in *Bacillus megaterium* [12]. Many bacteria can synthesize various types of PHAs containing 3-, 4-, and 5-hydroxyalkanoate units, and approximately 150 hydroxyalkanoates other than 3-hydroxybutyrate are known as constitutive units of PHAs [13]. PHA-producing microorganisms, such as *Ralstonia eutropha*, are used for the commercial production of PHAs, and their monomer composition, which is responsible for their mechanical properties, is regulated by varying carbon resources. However, because monomer composition is governed not only by the substrate specificities of monomer-supplying enzymes and PHA synthase but also by the combination of both wild-type enzymes, the monomer composition and the kind of unit in the polymer are limited to a certain extent [14, 15]. To overcome this problem, protein engineering of one of the monomer-supplying enzymes and three types of PHA synthases was performed [16, 17].

(*R*)-Specific enoyl-coenzyme A (CoA) hydratase (PhaJ), which is found in the β-oxidation pathway, catalyzes the stereoselective (*R*-specific) hydration of *trans*-2-enoyl-CoA to generate (*R*)-3-hydroxyacyl-CoA (3HA-CoA) for PHA biosynthesis. The crystal structure of $PhaJ_{Ac}$ derived from *Aeromonas caviae* was determined at a resolution of 1.5 Å (Protein Data Bank accession code 1IQ6) by Hisano *et al.* [18]. As shown in Fig. (**2**), the monomer structure of the enzyme consists of one five-strand antiparallel β-sheet and five α-helices. Two monomers associate to form a functional homodimer with an extended 10-strand β-sheet, and this structure is generally referred to as a "hotdog" fold. The catalytic residues, Asp31 and His36, are located deep in the substrate-binding pocket. Then, site-specific mutagenesis of $PhaJ_{Ac}$ was carried out to broaden substrate specificity [19]. Based on structural information, amino acid substitutions were introduced into $PhaJ_{Ac}$ by site-specific mutagenesis to Ser62, Leu65, and Val130, which are expected to affect the width and depth of the acyl-chain-binding pocket. Of the mutants generated, L65A, L65G, and V130G exhibited significantly high activities toward octenoyl-CoA compared to the wild-type enzyme. Thus, the acyl chain length substrate specificity of $PhaJ_{Ac}$ can be varied. This protein engineering study is the first to successfully achieve enzyme modification based on rational design.

PHA synthase (PhaC) catalyzes the polymerization of 3-HA-CoA monomers to PHAs and has been subjected to various forms of protein engineering to improve enzyme activity or substrate specificity. Unfortunately, the lack of a suitable structural model of

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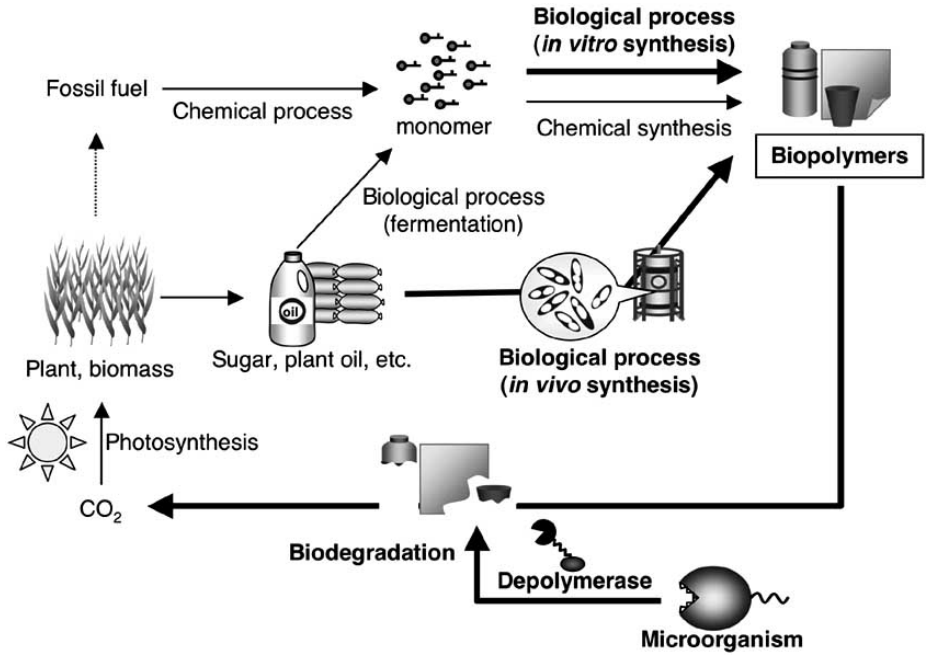

Fig. (1). Biopolymer recyclable ecosystem involving biological and/or chemical syntheses and biological degradation.

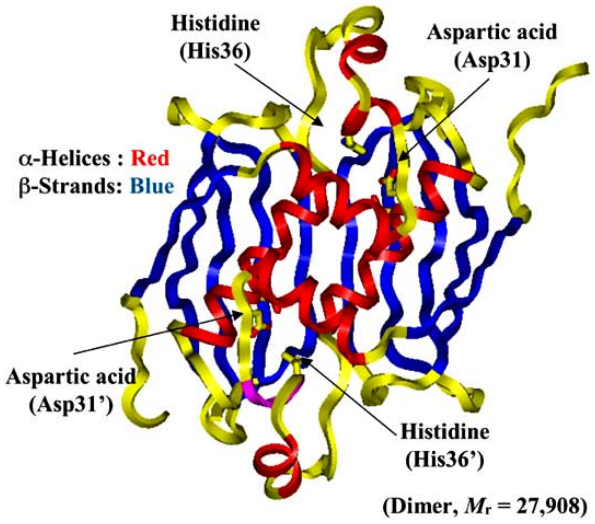

Fig. (2). Crystal structure of dimeric PhaJ determined at 1.5 Å resolution [18].

PHA synthase has limited attempts to improve activity and stability and to alter substrate specificity of the enzyme by "irrational" approaches, such as random mutagenesis and gene shuffling. Once function-related residues have been identified by these irrational approaches, site-specific saturation mutagenesis of residues known to affect the activity of the enzyme and the recombination of beneficial mutations can be used to improve the enzyme. Generally, natural diversity provides attractive starting materials for artificial evolution as it represents functionalized sequence spaces to some extent. The presence of a large population (over 60 species) of randomly screened PHA-producing bacteria suggests the existence of attractive prototype enzymes for molecular breeding. The enzyme evolution approach has been applied to type I and type II PHA synthases derived from bacteria, including *R. eutropha* (type I), *A. caviae* (type I), and *Pseudomonas* sp. 61-3 (type II). Through systematic directed evolution studies of PHA synthases [16, 17], we have acquired a huge library of evolved enzymes that can contribute to improving PHA production and dramatically changing monomer composition and molecular weight of PHA copolymer, which are closely related to material properties. Fig. (**3**) shows typical results of wide-range alterations of 3HB fraction and molecular weight of PHA copolymer, as observed in enzyme evolution studies of *Pseudomonas* sp. 61-3 PHA synthase. These successful results allow us to prepare desirable polymer materials using the best fine-tuned evolved enzyme in a tailor-made manner. These attractive mutant enzymes screened in the *E. coli* system are also applicable to other practical bacteria, such as *R. eutropha* [20-23] and *Corynebacterium glutamicum* [24, 25], and plants [26].

The pioneering study on chemo-enzymatic synthesis was conducted by Gerngross and Martin [27]. Granule formation of PHB was accomplished *in vitro* by combining purified type I PHA synthase from *R. eutropha* with synthetically prepared 3HB-CoA. This *in vitro* polymerization system produced PHB with an ultra-high molecular weight of 10 x 10^6 Da. The molecular weight can also be controlled by varying enzyme concentration. Based on the system that used substrate analogs, the CoA moiety was found to be essential for enzyme catalysis. Copolymers of 3HB with 3-hydroxyvalerate [28] and 3-hydroxypropionate [29] were synthesized *in vitro* using the same PHA synthase by other groups. The addition of glycerol to the reaction mixture stabilized PHA synthase and eliminated the lag phase in *in vitro* polymerization reactions. A similar *in vitro* polymerization of PHA was conducted using another type enzyme, type III PHA synthase from *Allochromatium vinosum*, and discussions about the chain termination mechanism in the polymer synthesis were reported [30, 31].

Jossek *et al*. reported a major breakthrough in these *in vitro* systems by recycling expensive CoA during the process of PHA polymerization [32, 33]. In combination with purified propionyl-CoA transferase from *Clostridium propionicum*, a two-enzyme *in vitro* polymerization system was established for PHB synthesis. The CoA residue for the activation of this hydroxy acid was provided by acetyl-CoA. By adding acetyl-CoA synthetase to this system, a three-enzyme *in vitro* PHB biosynthesis system was established. CoA that was released during the polymerization reaction coupled to acetate that again served as the CoA donor for the activation of

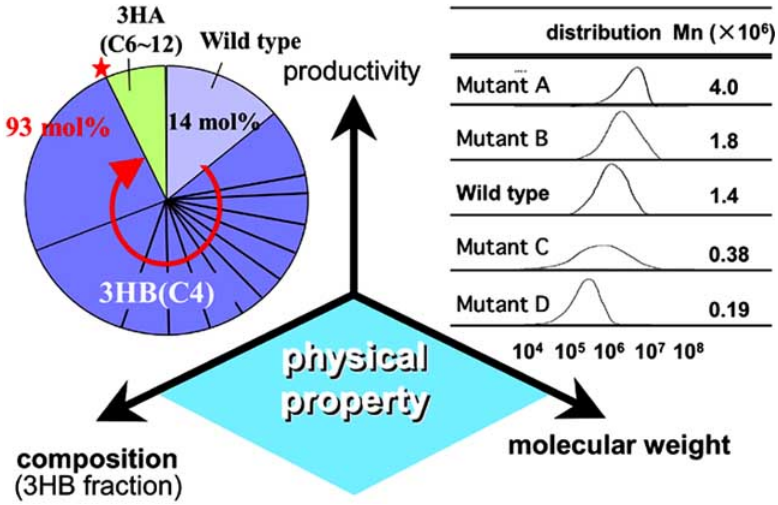

Fig. (3). Physical properties of the polymer regulated by varying monomer composition and molecular weight.

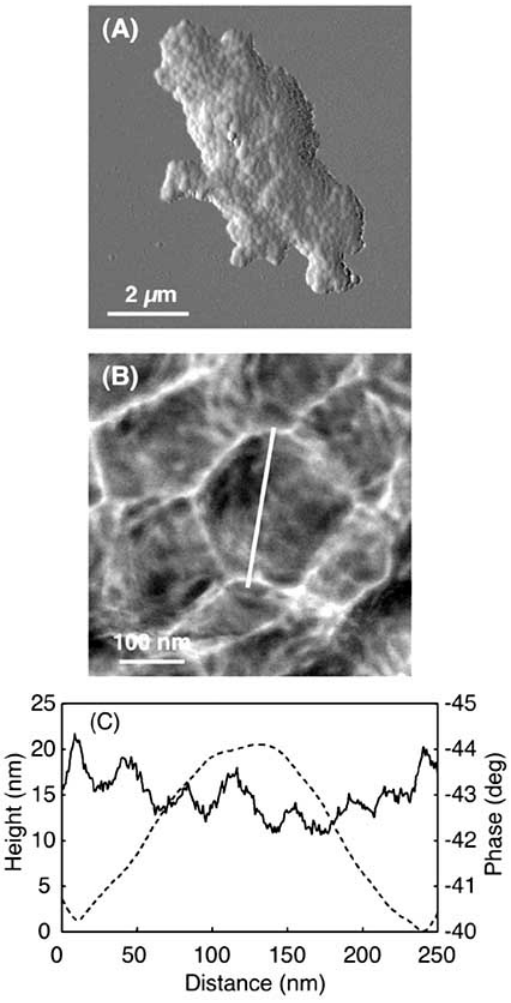

Fig. (4). (A) AFM amplitude image of PHB granule cluster synthesized *in vitro* at a reaction time of 10 min. (B) Enlarged AFM phase image from panel A. (C) Cross section along the white line indicated in panel B. Dotted and solid lines indicate height and phase profiles, respectively [37].

3HB. The energy for the *in vitro* PHB synthesis was provided by ATP hydrolysis, and this resulted in acetyl-CoA synthesis catalyzed by acetyl CoA synthetase. Based on the above system, Satoh *et al*. regenerated coenzyme NADPH for use as an energy donor in PHB and P(3HB-*co*-4HB) copolymerization by coupling with the CoA-recycling system [34, 35]. Furthermore, a water/organic solvent two-phase system was developed to generate one 3HA-CoA molecule via the exchange reaction of free CoA with 3HA-TP (thiophenyl ester) at the interface of the two phases [36]. This new system enables the non-enzymatic CoA transfer to 3HA to generate the monomer substrate precursor for PHA synthase.

Using atomic force microscopy (AFM), Hiraishi *et al*. presented direct evidence of granule formation during the course of PHB polymerization and proposed a possible mechanistic model of PHB polymerization [37]. Their results provide valuable new information and strongly support the mechanism of *in vitro* PHB polymerization proposed by Gerngross and Martin [27]. High-resolution AFM phase images of PHB granules provide direct information of the surface morphology of PHB granules (Fig. **4**), namely small protrusions corresponding to the apparent size of $PhaC_{Re}$ cover densely the surface of PHB granules.

ENZYMATIC DEGRADATION OF BIOPOLYESTERS

Biodegradability is considered to be one of the most important characteristics of biopolymers. After disposal, biopolymers are degraded in natural environments, such as soil, active sludge, fresh water, and seawater. Many prokaryotic and eukaryotic microorganisms secrete extracellular depolymerases capable of hydrolyzing biopolymers, and utilize the resultant decomposed compounds as nutrients. Finally, the compounds can be converted into renewable resources, such as CO_2 and biomass (Fig. **1**). Therefore, the decomposition of polymers to oligomers and/or monomers by depolymerases is important as the first step in biopolymer recycling. In regard to the stability of biopolymers and their controlled biodegradation after disposal, many researchers have investigated the biodegradation mechanisms of biopolymers, including polyesters, polysaccharides, proteins, lignins, and related polymers, from both physicochemical and biochemical aspects. In this section, we focus on the enzymatic degradation of biopolyesters, particularly PHAs, by extracellular PHA depolymerases.

A number of PHA depolymerases have been purified from diverse PHA-degrading microorganisms and characterized [8, 38, 39]. Among them, PHB depolymerases have been extensively examined. The purified enzymes are composed of a single polypeptide and their molecular weights are in the range of 37-60 kDa. Genetic analysis reveals that PHB depolymerases generally have a

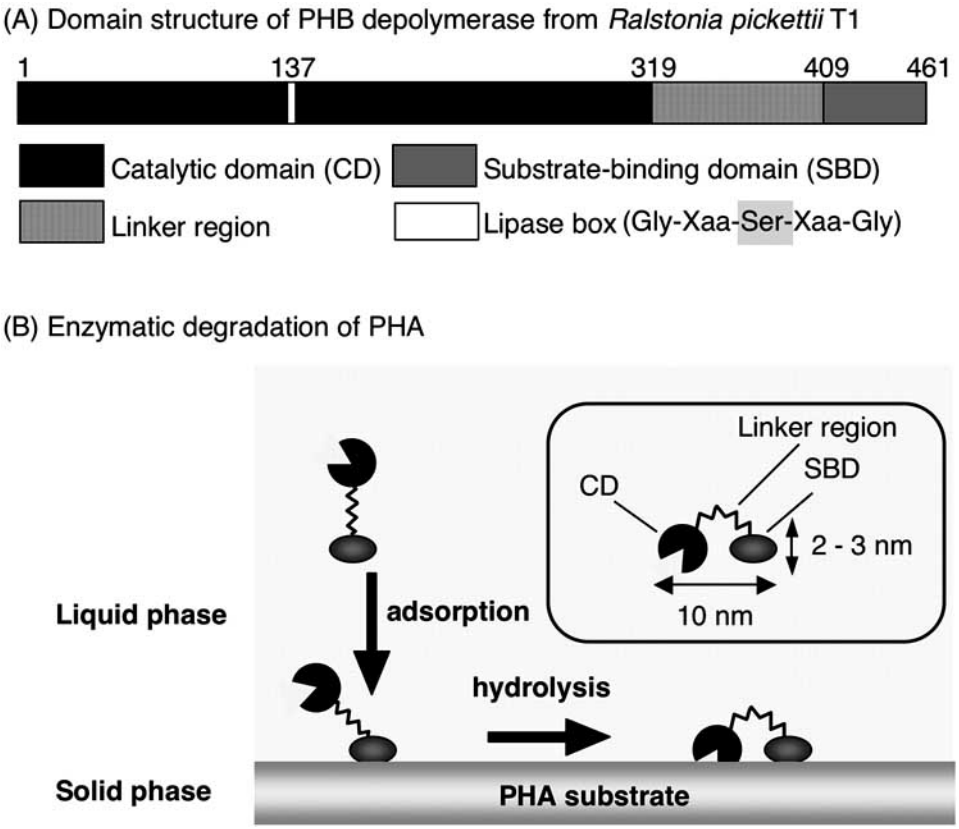

Fig. (5). (**A**) Domain structure of PHB depolymerase from *R. pickettii* T1. (**B**) Schematic illustration of the enzymatic degradation of polyester by PHB depolymerase.

domain structure consisting of a catalytic domain (CD) at N-terminus, a substrate-binding domain (SBD) at C-terminus, and a linker region connecting the two domains. For instance, the domain structure of PHB depolymerase from *Ralstonia pickettii* T1 is illustrated in Fig. (**5**(**A**)). Such domain structure has been found in many biopolymer-degrading enzymes, such as cellulase [40], xylanase [41], and chitinase [42], which are capable of hydrolyzing water-insoluble polysaccharides. Genetic analysis also shows that PHB depolymerases contain a lipase box pentapeptide [Gly-X_1-Ser-X_2-Gly] as an active center, indicating that these enzymes are one of the serine hydrolases. The enzymatic degradation of PHA by PHB depolymerases is considered to proceed via a two-step reaction at the solid-liquid interface, as shown in Fig. (**5**(**B**)). PHB depolymerase approaches and adheres to the polymer surface via SBD, and this is followed by hydrolysis of the polymer chain by CD. Accordingly, it is considered that elucidation of the mechanisms of enzyme adsorption and enzymatic hydrolysis will contribute to the development of new polymer materials with the desired stability and biodegradability as well as optimal structure and physical properties.

The effects of the chemical structures of PHAs on enzymatic hydrolysis have been investigated. For this purpose, various types of PHAs, such as racemic PHA [43-50] and 3HA oligomers [51, 52], PHAs with different main- and side-chain lengths [53], and random copolymers of (*R*)-3HB with various hydroxyalkanoate units [54-57], have been synthesized for use in enzymatic degradation by a variety of PHB depolymerases. Based on the results of those studies, a schematic model of the enzymatic cleavage of the PHA chain by PHB depolymerase from *R. pickettii* T1 is proposed, as shown in Fig. (**6**) [55]. The active site in PHB depolymerase CD recognizes at least three neighboring monomer units in the polymer chain as substrate. Besides the effects of the chemical structure, the effects of solid-state structure and surface properties of PHAs on the enzymatic hydrolysis have also been investigated. For instance, the amorphous regions in PHA materials are preferentially hydrolyzed, so that the hydrolysis of crystalline regions is the rate-limiting step in the enzymatic degradation process [58, 59]. Further, the enzymatic degradation rate of PHA materials decreases with increasing crystallinity, crystal size, and regularity of the chain packing state. Abe and co-workers demonstrated that the change of the surface properties of poly(lactic acid) induced by end-capping with alkyl ester groups (carbon numbers 12 to 14) leads to a decrease in the enzymatic degradation rate of the end-capped polymers [60, 61].

To investigate the influence of the chemical structure or surface properties of polymer on enzymatic adsorption at nano-level sensitivity, several studies have been performed using quartz crystal microbalance (QCM) and AFM. Yamashita *et al*. studied the adsorption of PHB depolymerase to the surface of several polymers, such as polyethylene, polystyrene, and PHA, by the QCM technique, and found that the enzyme has adsorption specificity for PHA [62-64]. In addition, AFM analysis of PHB depolymerase on polyester surface has revealed that small ridges are formed around the enzyme molecule due to movement of some polyester chains at the adsorption area, suggesting that a strong chemical interaction exists between the enzyme and the polyester chains [64, 65]. Furthermore, AFM analysis of PHB single crystal and a hydrolytic-activity-disrupted mutant of PHB depolymerase has demonstrated that the SBD of PHB depolymerase disturbs the molecular packing of PHB polymer chains, resulting in fragmentation of the PHB single crystal [66]. Similarly, in the enzymatic degradation of cellulose by cellulase [67] or chitin by chitinase, [68] several researchers have reported that the binding domain of these enzymes enhances the specific physical disruption of their substrates. Taking these

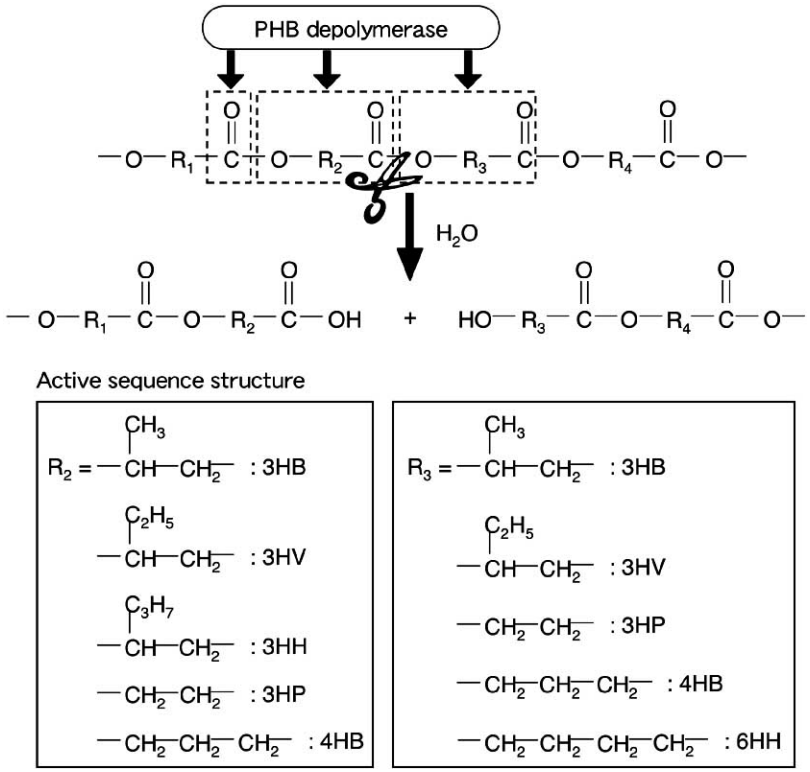

Fig. (6). Schematic model of enzymatic cleavage of an ester bond in various sequences by PHB depolymerase [55].

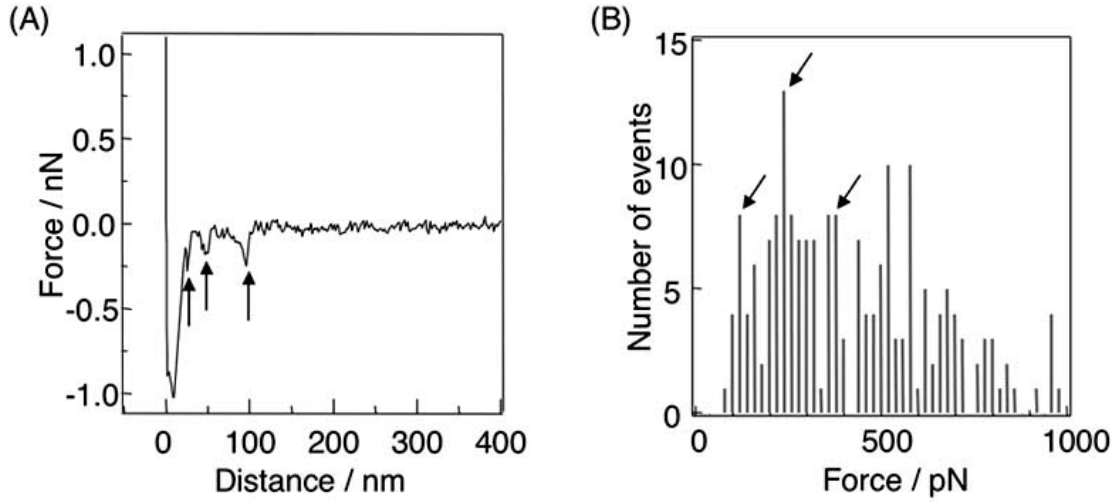

Fig. (7). (**A**) Typical retraction curve of force-distance measurements of PHB thin film using AFM tip functionalized with His-tagged SBD. Arrows indicate several minima exhibited in the retraction curve. (**B**) Histogram of pull-off events obtained in the force-distance measurement between PHB and His-tagged SBD tip. Bin size is 20 pN [69].

findings into consideration, the specific adsorption of PHB depolymerase to the PHB surface probably involves both the adsorption of the enzyme to the surface and the non-hydrolytic disruption of the substrate to promote PHB degradation. Recently, we have developed the AFM technique by using an AFM tip modified with SBD protein to evaluate the interaction between the SBD molecule and the PHB surface at the molecular level. Through this, we found that the adsorption force of one SBD molecule to the PHB surface is approximately 100 pN (Fig. **7**) [69].

From a biological viewpoint, the structure-function relationship of PHB depolymerases has been studied extensively, and several mutants were designed to analyze the function of each domain, in particular, SBD. Using a truncated PHB depolymerase, Behrends *et al.*, Nojiri and Saito, and our group revealed that the C-terminal domain is essential for PHB-specific binding [70-72]. Further, Nojiri and Saito genetically prepared many mutants of the *R. pickettii* T1 enzyme in various forms including inversions, chimeras, and fusion to extra linker domains [71]. Their results suggest that SBD organization in the enzyme also influences the degradation of PHB but not water-soluble substrates. Doi and co-workers prepared fusion proteins of SBDs of several PHB depolymerases with glutathione *S*-transferase [73-77], and demonstrated specific interactions based on molecular recognition between SBD and polyester surface.

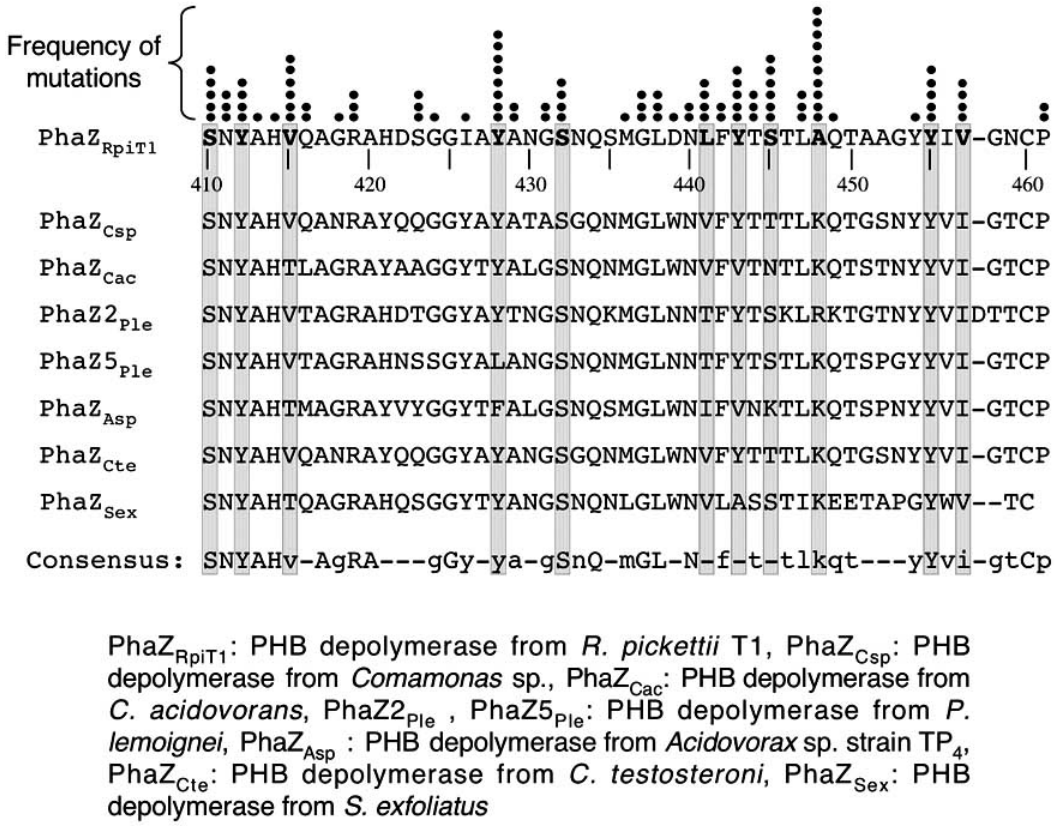

Fig. (8). Positions and frequencies of PCR-mediated single mutations in the region coding for SBD of PHB depolymerase from *R. pickettii* T1, together with alignment of SBDs of other PHB depolymerases. Amino acid residues conserved among PHB depolymerases are indicated by capital letters in the consensus sequence; those which have been conserved in six or more proteins are marked by small letters in the consensus sequence. Amino acids that were substituted with other residues at high frequencies in $PhaZ_{RpiT1}$ mutants are shaded [78].

Thus far, there have been only a few attempts to determine which amino acid residues in PHB depolymerases participate in the enzymatic adsorption to PHB surface, and how these amino acids contribute to the enzymatic adsorption. We have investigated the interaction between PHB depolymerase from *R. pickettii* T1 and PHB surface by a combination of PCR-mediated random mutagenesis targeted to only SBD and an *in vivo* screening system [78]. Genetic analysis of the isolated mutants with lowered activity showed that Ser, Tyr, Val, Ala, and Leu residues in SBD were substituted by other residues at high frequency (Fig. **8**). The results suggested that PHB depolymerase adsorbs to the PHB surface not only via the formation of hydrogen bonds between hydroxyl groups of Ser in the enzyme and carbonyl groups in the PHB polymer, but also via the hydrophobic interaction between hydrophobic residues in the enzyme and methyl groups in the PHB polymer. In addition to PHB depolymerase, the functional analysis of poly(3-hydroxyoctanoate) (PHO) depolymerase from *Pseudomonas fluorescens* GK13 was carried out using similar random mutagenesis techniques by Jendrossek *et al*. [79]. PHO depolymerase is specific for PHA having medium chain length, and its amino acid sequence shows no typical domain structures and no similarity to those of other PHA depolymerases. They reported that Ser17, Phe50, and Phe63 in the N-terminal region of PHO depolymerase are possibly involved in the interaction between the enzyme and the PHO polymer surface. Recently, Hisano *et al*. determined the crystal structure of single-domain PHB depolymerase from *Penicillium funiculosum* [80]. They proposed that hydrophobic residues, including Tyr, Leu, Ile, and Val, contribute to adsorption to the PHB surface, and that hydrophilic residues (Ser and Asn) located around the mouth of the enzyme crevice may also contribute to the affinity of the enzyme for PHB.

Based on the results of functional and structural analyses of PHB depolymerases as mentioned above and the chemical structure of PHB containing carbonyl and methyl groups as functional groups, we have proposed a plausible model of the interaction between the SBD of PHB depolymerase from *R. pickettii* T1 and the PHB surface (Fig. **9**) [78]. It is reported that an interactive force of approximately 100 pN exists between the SBD of PHB depolymerase and the PHB polymer surface [69]. This interaction involves not only hydrogen bonding between hydrophilic residues such as Ser in the enzyme and ester bonds in the polymer, but also hydrophobic interactions between hydrophobic residues such as Leu and Val in the enzyme and methyl groups in the polymer. As a result, SBD probably plays a key role not only in the specific adsorption of PHB depolymerase to the PHB surface but also in the non-hydrolytic disruption of the substrate to promote PHB degradation.

IN VITRO ENZYME-CATALYZED SYNTHESIS OF BIO-POLYESTERS

Enzymes possess such advantages as high catalytic rates, lack of undesirable by-products, high enantio- and regioselectivities, and the ability to function under mild conditions, in comparison with other chemical catalysts [81, 82]. These characteristics have initiated studies of *in vitro* synthesis (via non-metabolic pathways) and modification of polymers as well as small compounds in recent years [83-85]. Furthermore, because enzymes are derived from renewable resources and are applicable to aqueous, low solvent or solventless systems, enzymatic polymerization using renewable resources as starting material has great potential as an environmentally compatible process. Enzymes used for *in vitro* polymer production are listed in Table **1**. Target polymers of *in vitro* enzymatic

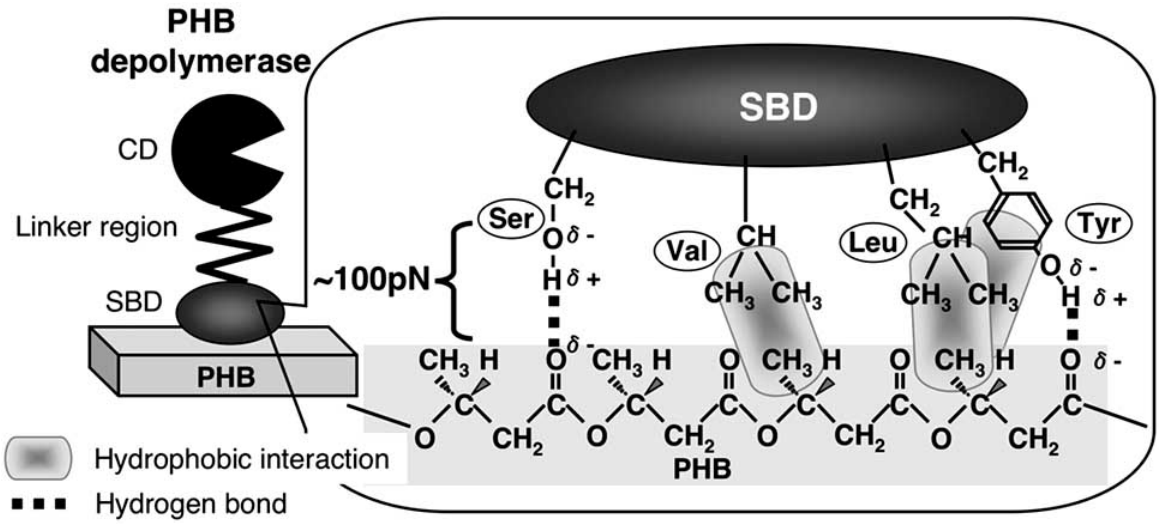

Fig. (9). Schematic illustration of the interaction between amino acid residues in SBD of PHB depolymerase from *R. pickettii* T1 and PHB polymer chain [78].

Table 1. Enzymes and Typical Polymers Produced *in Vitro* by Respective Enzymes

Enzyme	Typical Polymer
Oxidoreductases	Polyphenols, polyanilines, vinyl polymers
Transferases	Polysaccharides, cyclic oligosaccharides
Hydrolases	Polysaccharides, poly(amino acid)s, polyamides, polyesters, polycarbonates
Isomerases	
Lyases	
Ligases	Polyesters

polymerization include polyesters, poly(amino acid)s, polysaccharides, polycarbonates, and so on.

It is generally accepted that an enzymatic reaction is substantially reversible and its equilibrium can be regulated by altering the reaction conditions. On the basis of these concepts, hydrolases are most commonly used for the enzyme-catalyzed synthesis of polyesters in organic solvents. Among the hydrolases used for the synthesis, lipases have been extensively studied because they possess broad substrate specificity and are occasionally stable in organic solvents (Table **1**). The lipase-catalyzed synthesis of polyesters has been performed since the 80s. Typical reactions of lipase-catalyzed synthesis of polyesters, i.e., polycondensation and ring-opening polymerization, are summarized in Fig. (**10**). The enzymatic polycondensation of hydroxy acids by lipase was first reported in the mid 80s, but the resultant products were oligomeric esters [86, 87]. Later, high molecular weight polyesters were synthesized from dicarboxylic acid esters and glycols. In 1993, ring-opening polymerization catalyzed by lipases was reported for the first time by Uyama and Kobayashi [88] and Knai *et al.* [89]. Uyama and Kobayashi performed the enzyme-catalyzed polymerization of δ-valerolactone (δ-VL) and ε-caprolactone (ε-CL) by using three kinds of commercially available lipases. Knai *et al.* reported the ring-opening polymerization of ε-CL around the same time. Thereafter, the lipase-catalyzed polymerization of various cyclic compounds has been investigated and lactones, in particular, have been extensively used as monomer substrate.

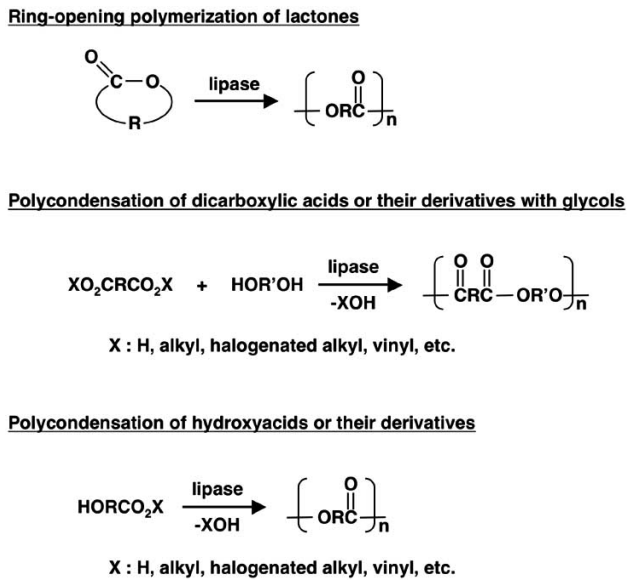

Fig. (10). Typical reaction types of lipase-catalyzed polymerization leading to polyesters.

The ring-opening polymerization of lactones by lipases is considered to proceed via an activated-monomer mechanism, as shown in Fig. (**11**) [90]. Lipases contain Ser residue as the catalytic site to form a complex with the lactone, resulting in an acyl-enzyme intermediate (enzyme-activated monomer). The intermediate reacts with water or alcohol to generate the enzyme and ω-hydroxycarboxylic acid or ester. In the propagation step, the terminal hydroxyl group of the propagating polymer nucleophilically attacks the intermediate, resulting in the addition of one more unit to the chain and enzyme regeneration.

In the lipase-catalyzed ring-opening polymerization, a large amount of enzyme is generally used because of the low polymerization rate. By contrast, *Candida antarctica* lipase (lipase CA) immo-

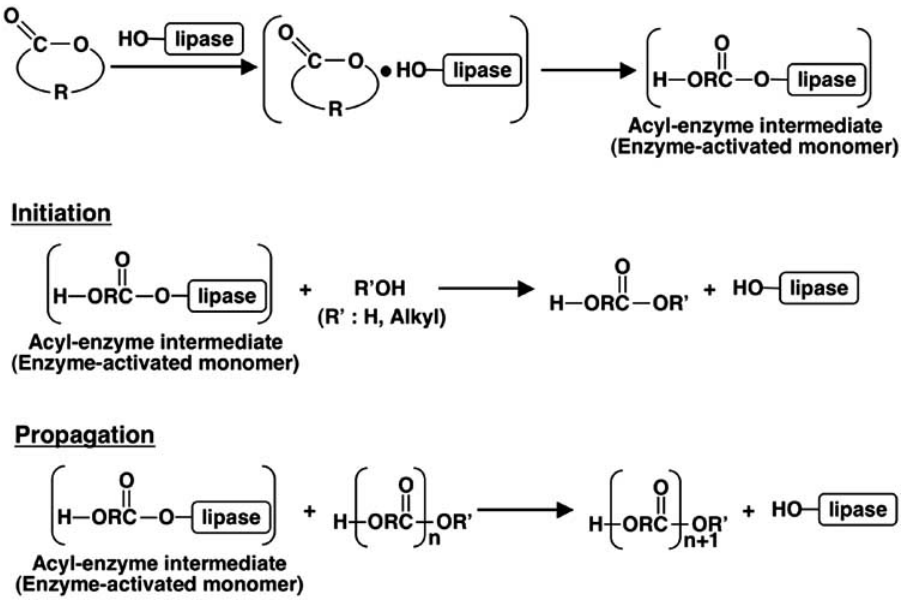

Fig. (11). Proposed mechanism of lipase-catalyzed polymerization of lactones [90].

Table 2. Dipole Moments and Reactivities of Various Unsubstituted Lactones for Lipase PL [97]

Lactone	Dipole Moment (μ) (D)	Relative Rate of Polymerization	Relative Rate of Polymerization
		Enzymatic Polymerization[a)]	Anionic Polymerization[b)]
δ-VL (6)	4.22	-	2,500
ε-CL (7)	4.45	0.10	330
8-OL (9)	2.25	-	21
UDL (12)	1.86	0.13	0.9
DDL (13)	1.86	0.19	1.0
PDL (16)[b)]	1.86	0.74	0.9
HDL (17)	-	1.00	1.0

δ-VL: δ-valerolactone, ε-CL: ε-caprolactone, 8-OL: 8-octanolide, UDL: 11-undecanolide, DDL: 12-dodecanolide, PDL: 15-pentadecanolide, HDL: 16-hexadecanolide

[a)] The polymerization was performed using lipase PF (200 mg) in the presence of 1-octanol in diisopropyl ether (10 mL) at 60 ˚C.

[b)] $[Zn(Oct)_2]_0=[BuOH]_0=0.28$ mol/L with bulk polymerization performed at 100 ˚C.

bilized on polymeric support has shown high performance for the ring-opening polymerization of lactones [91]. Lipase CA immobilized on macroporous acrylic resin (trade name: Novozym® 435) has exceptionally high catalytic activity: a small amount of this lipase (less than 1%) could induce the ring-opening polymerization of ε-CL, and its molecular weight exceeded 4×10^4 under optimum conditions [92, 93]. Further, Novozym® 435 could be used repeatedly for the synthesis of poly(ε-CL), and the polymerization activity remained unchanged up to five cycles [94]. Uyama *et al*. demonstrated that lipase CA immobilized on polypropylene has high activity for the polymerization of 15-pentadecanolide (PDL) macrolide compared with Novozym® 435 [91]. Besides the high catalytic activity of lipase CA, its versatility has led to use in the synthesis or chemical recycling of polycarbonates and poly(ester-urethane)s as well as polyesters [95, 96].

The lipase-catalyzed ring-opening polymerization of various lactones, including substituted and/or unsubstituted ones, has been reported. Reactivity of the lactones is generally considered to depend on ring strain and basicity. Unsubstituted lactones with ring size of 4 to 17 have been used for the lipase-catalyzed ring-opening polymerization. Kobayashi investigated the reactivity of lactones with different ring sizes in the ring-opening polymerization by lipase PF from *Pseudomonas fluorescens* and compared it to that in chemical polymerization [97]. Table **2** summarizes dipole moments and reactivities of the lactones. The dipole moments of the monomers can be taken as an indication of ring strain, and small and medium-sized lactones show higher dipole moments than large ones (macrolides). In the chemical reaction, small and medium-sized lactones with high dipole moments (indicative of large strain) showed higher polymerization rates than large lactones with low dipole moments. In contrast, macrolide monomers (10- to 17-membered) had higher reactivity for ring-opening polymerization by lipase PF than medium-sized monomers (6- and 7-membered). Meijer and co-workers analyzed the kinetics of Novozym® 435-catalyzed ring-opening polymerization of lactones with various ring sizes (6- to 13- and 16-membered) and demonstrated fascinating differences in polymerization rates (Table **3**) [98].

A variety of substituted lactones have been prepared and applied to lipase-catalyzed ring-opening polymerization. For example, PHB [99-101] and poly(malic acid) derivative [poly(benzyl β-malolactonate)] [102] were enzymatically synthesized from β-butyrolactone (β-BL) and benzyl β-malolactonate (4-membered lactones), respectively. Kobayashi and co-workers reported the ring-opening polymerization of α-methyl-substituted medium-sized lactones, α-methyl-δ-VL and α-methyl-ε-CL, in bulk by using lipase CA as catalyst [103]. They further systematically investigated the effects of substituted position and ring size of methyl-substituted lactones in the lipase-CA-catalyzed polymerization [104]. In the case of 6- and 7-membered lactones, the reaction behaviors of α-substituted lactones and γ-methyl-ε-CL were relatively

Table 3. Dipole Moments and Michaelis-Menten Constants of Lipase CA (Novozym® 435) in Ring-Opening Polymerization of Lactones at 45˚C [98]

Lactone	Dipole Moment (μ) (D)	K_m (mol/L)	V_{max} (mol/L·h)	k_{cat} [a] (s^{-1})	k_{cat}/K_m (L/(mol · s))
δ-VL (6)	4.22	0.73	0.95	35.2	48.2
ε-CL (7)	4.45	0.72	1.97	72.9	101.3
7-HL (8)	3.70	0.09	6.10	225.9	510.3
8-OL (9)	2.25	0.16	1.29	47.7	298.6
9-NL (10)	2.01	0.11	0.07	2.6	23.6
DL (11)	1.88	0.40	0.11	4.0	10.2
UDL (12)	1.86	0.33	0.37	13.7	41.5
DDL (13)	1.86	0.42	2.80	103.7	246.9
PDL (16) [b]	1.86	0.31	5.51	204.0	658.9

δ-VL: δ-valerolactone, ε-CL: ε-caprolactone, 8-OL: 8-octanolide, 9-NL: 9-nonanolactone, DL: 10-decanolactone, UDL: 11-undecanolide, DDL: 12-dodecanolide, PDL: 15-pentadecanolide

[a] $[\text{lipase CA}]_{tot}$ = 7.5 μM. [b] measured at 60 ˚C.

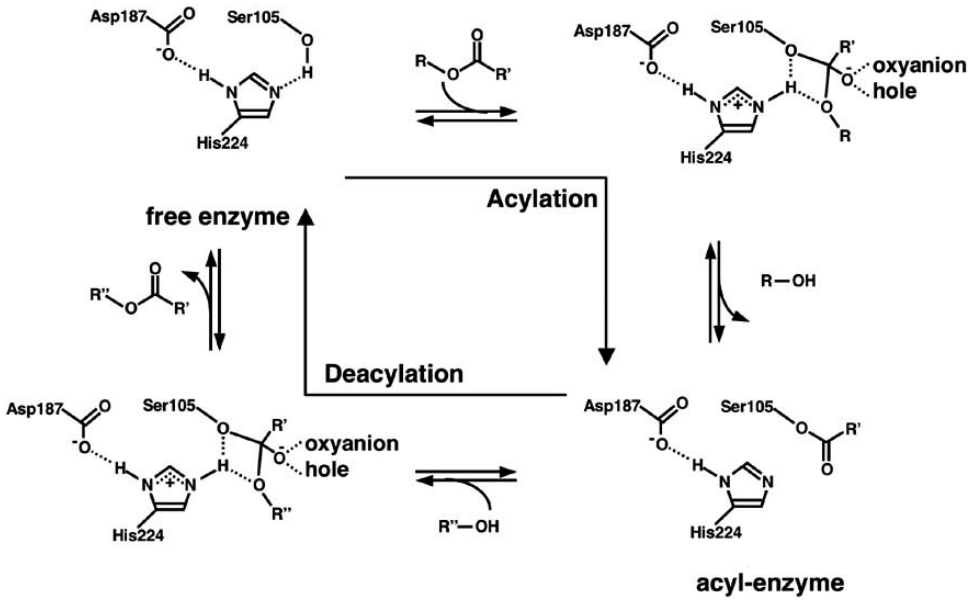

Fig. (12). Reaction mechanism of lipase CA [107].

similar to those of unsubstituted ones, while the polymerizability of α-methyl-substituted macrolides (13- and 16-membered) decreased with the introduction of a methyl substituent. Haufe and co-workers reported that fluorinated lactones (medium-sized and large lactones) were polymerized by lipase CA [105].

The availability of inexpensive and enantiomerically pure monomers has played a key role in the development of the synthesis of chiral polymers, as most monomers are not easily available in enantiopure form. Thus, the enantioselective polymerization of substituted racemic lactones by lipases has been investigated in order to dispense with the need for enantiopure monomers. The enantioselective polymerization of four-membered lactones, α-methyl-β-propiolactone and β-butyrolactone, was performed by Gross and co-workers [106] and Wang and co-workers [100], respectively. A range of chiral polyesters were synthesized using substituted racemic ε-CLs as monomers via enantioselective ring-opening polymerization by lipases; in particular, Novozym® 435 was employed. The enantiomeric excess (ee) of the polymer was generally moderate in the enantioselective polymerization of substituted racemic ε-CL, and was related to the moderate selectivity of Novozym® 435 for the substrates. Meijer and co-workers [107] and Bisht and co-workers [108] demonstrated that highly (*S*)-enriched substituted poly(ε-CL)s could be synthesized from 4-methyl- and 4-ethyl-ε-CLs, and (*R*)-enriched one, from 4-propyl-ε-CL. The action of lipase CA proceeds via two steps: acylation and deacylation, which are separated by a covalent acyl-enzyme intermediate, as shown in Fig. (**12**), in which Ser105, His224, and Asp187 constitute the catalytic triad as an active site. Their results demonstrate that for 4-substituted ε-CLs, the chirality of the propagating alcohol chain end is important in the catalytic cycle, and the rate-limiting step is more likely the deacylation of the acyl-enzyme intermediate by the propagating alcohol chain end, but not necessarily the formation of the intermediate. Furthermore, Meijer and co-workers comprehensively studied the Novozym® 435-catalyzed ring-opening polymerization of ω-methylated lactones with ring sizes of 4 to 13 [109]. They successfully synthesized (*R*)-enriched poly(ε-CL)s from substituted lactones with ring sizes ≥ 8, and the synthesized polymers have the high ee values (>99%) obtained for lipase-CA-catalyzed ring-opening polymerization of racemic lactones.

CONCLUSION

This mini-review describes various polymerization systems of biopolyesters as well as the enzymatic degradation by their depolymerases. Biopolyesters present a multitude of benefits as sub-

stitutes for conventional polymers synthesized from fossil fuels. Among them, PHAs are one of the desirable alternatives to petrochemical-derived polymers because PHAs are produced directly from renewable resources and can be renewed over a short time. However, three main issues have hindered widespread use: the high production cost compared to petroleum-based polymers with similar properties; the inability to produce high-performance PHA polymers in substantial amounts; and the difficulty in controlling the life cycle of PHA polymers, i.e., the control of their biodegradability. Breakthroughs in the genetic engineering of metabolic pathways have led to the cost-effective biological production of PHAs and the improvement of their properties, such as molecular weight and monomer composition. In particular, engineering PHA synthase can improve both PHA production efficiency and the properties of the generated polymer because PHA synthase plays a central role in PHA biosynthesis. Further, gene cloning and expression in plants has created new possibilities of using photosynthesis to convert atmospheric CO_2 directly into PHA, in hopes of reducing production cost in the future.

A separate move is the use of isolated enzymes that have found increasing application as catalysts for biopolymer synthesis *in vitro*, because of their high catalytic rates, lack of undesirable by-products, high enantio- and regioselectivities, and ability to function under mild conditions. Enzymatic polymerization *in vitro* offers a number of advantages, including easier control of polymer structure and monomer reactivity than conventional chemical methods in which extensive multistep protection-deprotection schemes are often required. In addition to the above advantages, enzymes are themselves part of a sustainable system and the polymer products synthesized via enzyme catalysis are, in most cases, biodegradable. Furthermore, from the viewpoint of preserving the ecosystem, biopolymers are most beneficial when they can be actually biodegraded. Accordingly, it is vital to elucidate the biodegradation mechanism of biopolymers, engineer their depolymerases, and develop *in vivo* and *in vitro* biosyntheses of biopolymers. In the future, custom-made prominent enzymes generated via evolutionary engineering will be utilized extensively to create high-performance biopolymers from renewable carbon sources in various organisms as well as improved *in vitro* systems.

REFERENCES

[1] Gross, R. A.; Kalra, B. *Science*, **2002**, *297*, 803.
[2] Gross, R. A.; Kalra, B. *Science*, **2003**, *299*, 822.
[3] Doi, Y.; Fukuda, K. Eds. *Biodegradable Plastics and Polymers*, Elsevier Science, Amsterdam, **1994**.
[4] Chiellini, E.; Solaro, R. Eds. *Biodegradable Polymers and Plastics*, Kluwer Academic/Plenum Publishers, New York, **2003**.
[5] Steinbüchel, A. Eds. *Biopolymers General Aspects and Special Applications*, Wiley-VCH, Weinheim, **2004**, vol. 10.
[6] Scholz, C.; Gross, R.A. Eds. *Polymers from Renewable Resources-Biopolyesters and Biocatalysis*, ACS Symposium Series 764; American Chemical Society, Washington, DC, **2000**.
[7] Doi, Y.; Steinbüchel, A. Eds. *Biopolymers Polyesters I: Biological Systems and Biotechnological Production*, Weinheim, Wiley-VCH, **2002**, vol. 3a.
[8] Doi, Y.; Steinbüchel, A. Eds. *Biopolymers Polyesters II: Properties and Chemical Synthesis*, Wiley-VCH, Weinheim, **2002**, vol. 3b.
[9] Sudesh, K.; Abe, H.; Doi, Y. *Prog. Polym. Sci.*, **2000**, *25*, 1503.
[10] Lenz, R. W.; Marchessault, R. H. *Biomacromolecules*, **2005**, *6*, 1.
[11] Doi, Y.; Steinbüchel, A. Eds. *Biopolymers Polyesters III: Applications and Commercial Products*, Wiley-VCH, Weinheim, **2002**, vol. 4.
[12] Lemoigne, M. *Ann. Inst. Pasteur*, **1925**, *39*, 144.
[13] Steinbüchel, A.; Valentin, H. E. *FEMS Microbiol. Lett.*, **1995**, *128*, 219.
[14] Aldor, I. S.; Keasling, J. D. *Curr. Opin. Biotechnol.*, **2003**, *14*, 475.
[15] Suriyamongkol, P.; Weselake, R.; Narine, S.; Moloney, M.; Shah, S. *Biotechnol. Adv.*, **2007**, *25*, 148.
[16] Taguchi, S.; Doi, Y. *Macromol. Biosci.*, **2004**, *4*, 145.
[17] Nomura, C.; Taguchi, S. *Appl. Microbiol. Biotechnol.*, **2007**, *73*, 969.
[18] Hisano, T.; Tsuge, T.; Fukui, T.; Iwata, T.; Miki, K.; Doi, Y. *J. Biol. Chem.*, **2003**, *278*, 617.
[19] Tsuge, T.; Hisano, T.; Taguchi, S.; Doi, Y. *Appl. Environ. Microbiol.*, **2003**, *69*, 4830.
[20] Taguchi, S.; Nakamura, H.; Kichise, T.; Tsuge, T.; Yamato, I.: Doi, Y. *Biochem. Eng. J.*, **2003**, *16*, 107.
[21] Tsuge, T.; Yano, K.; Imazu, S.; Numata, K.; Kikkawa, Y.; Abe, H.; Taguchi, S.; Doi, Y. *Macromol. Biosci.*, **2005**, *5*, 112.
[22] Tsuge, T.; Watanabe, S.; Saito, S.; Hiraishi, T.; Abe, H.; Doi, Y.; Taguchi, S. *Macromol. Biosci.*, **2007**, *7*, 846.
[23] Tsuge, T.; Watanabe, S.; Shimada, D.; Abe, H.; Doi, Y.; Taguchi, S. *FEMS Microbiol. Lett.*, **2007**, *277*, 217.
[24] Jo, S.-J.; Maeda, M.; Ooi, T.; Taguchi, S. J. *Biosci. Bioeng.*, **2006**, *102*, 233.
[25] Jo, S.-J.; Matsumoto, K.; Leong, C. R.; Ooi, T.; Taguchi, S. *J. Biosci. Bioeng.*, **2007**, *104*, 457.
[26] Matsumoto, K.; Arai, Y.; Nagao, R.; Murata, T.; Takase, K.; Nakashita, H.; Taguchi, S.; Shimada, H.; Doi, Y. *J. Polym. Environ.*, **2006**, *14*, 369.
[27] Gerngross, T. U.; Martin, D. P. *Proc. Natl. Acad. Sci. USA*, **1995**, *92*, 6279.
[28] Lenz, R. W.; Farcet, C.; Dijkstra, P. J.; Goodwin, S.; Zhang, S. M. *Int. J. Biol. Macromol.*, **1999**, *25*, 55.
[29] Song, J. J.; Zhang, S.; Lenz, R. W.; Goodwin, S. *Biomacromolecules*, **2000**, *1*, 433.
[30] Zhang, S.; Kolvek, S.; Goodwin, S.; Lenz, R. W. *Biomacromolecules*, **2004**, *5*, 40.
[31] Lawrence, A. G.; Choi, J.; Rha, C.; Stubbe, J.; Sinskey, A. J. *Biomacromolecules*, **2005**, *6*, 2113.
[32] Jossek, R.; Steinbüchel, A. *FEMS Microbiol. Lett.*, **1998**, *168*, 319.
[33] Jossek, R.; Reichelt, R.; Steinbüchel, A. *Appl. Microbiol. Biotechnol.*, **1998**, *49*, 258.
[34] Satoh, Y.; Tajima, K.; Tannai, H.; Munekata, M. *J. Biosci. Bioeng.*, **2003**, *95*, 335.
[35] Satoh, Y.; Murakami, F.; Tajima, K.; Munekata, M. *J. Biosci. Bioeng.*, **2005**, *99*, 508.
[36] Tajima, K.; Satoh, Y.; Nakazawa, K.; Tannai, H.; Erata, T.; Munekata, M.; Kamachi, M.; Lenz, R. W. *Macromolecules*, **2004**, *37*, 4544.
[37] Hiraishi, T.; Kikkawa, Y.; Fujita, M.; Normi, Y. M.; Kanesato, M.; Tsuge, T.; Sudesh, K.; Maeda, M.; Doi, Y. *Biomacromolecules*, **2005**, *6*, 2671.
[38] Jendrossek, D.; Handrick, R. *Annu. Rev. Microbiol.*, **2002**, *56*, 403.
[39] Kim, D. Y.; Rhee, Y. H. *Appl. Microbiol. Biotechnol.*, **2003**, *61*, 300.
[40] Gilkes, N. R.; Henrissat, B.; Kilburn, D. G.; Miller, R. C. J.; Warren, R. A. J. *Microbiol. Rev.*, **1991**, *55*, 303.
[41] Kellet, L. E.; Poole, D. M.; Ferreira, L. M. A.; Durrant, A. J.; Hazlewood, G. P.; Gilbert, H. J. *Biochem. J.*, **1990**, *272*, 369.
[42] Watanabe, T.; Suzuki, K.; Oyanagi, W.; Ohnishi, K.; Tanaka, H. *J. Biol. Chem.*, **1990**, *265*, 659.
[43] Kemnitzer, J. E.; McCarthy, S. P.; Gross, R. A. *Macromolecules*, **1992**, *25*, 5927.
[44] Jesudason, J. J.; Marchessault, R. H.; Saito, T. *J. Environ. Polym. Degrad.*, **1993**, *1*, 89.
[45] Hocking, P. J.; Marchessault, R. H. *Polym. Bull.*, **1993**, *30*, 163.
[46] Abe, H.; Matsubara, I.; Doi, Y.; Hori, Y.; Yamaguchi, A. *Macromolecules*, **1994**, *27*, 6018.
[47] Hocking, P. J.; Timmins, M. R.; Scherer, T. M.; Fuller, R. C.; Lenz, R. W.; Marchessault, R. H. *Macromol. Rapid Commun.*, **1994**, *15*, 447.
[48] Hocking, P. J.; Timmins, M. R.; Scherer, T. M.; Fuller, R. C.; Lenz, R. W.; Marchessault, R. H. *J. Macromol. Sci. Pure Appl. Chem. A*, **1995**, *32*, 889.
[49] Timmins, M. R.; Lenz, R. W.; Hocking, P. J.; Marchessault, R. H.; Fuller, R. C. *Macromol. Chem. Phys.*, **1996**, *197*, 1193.
[50] Abe, H.; Doi, Y. *Macromolecules*, **1996**, *29*, 8683.
[51] Bachmann, B. M.; Seebach, D. *Macromolecules*, **1999**, *32*, 1777.
[52] Scherer, T. M.; Fuller, R. C.; Goodwin, S.; Lenz, R. W. *Biomacromolecules*, **2000**, *1*, 577.
[53] Kasuya, K.; Ohura, T.; Masuda, K.; Doi, Y. *Int. J. Biol. Macromol.*, **1999**, *24*, 329.
[54] Kanesawa, Y.; Tanahashi, N.; Doi, Y.; Saito, T. *Polym. Degrad. Stab.*, **1994**, *45*, 179.
[55] Abe, H.; Doi, Y.; Aoki, H.; Akehata, T.; Hori, Y.; Yamaguchi, A. *Macromolecules*, **1995**, *28*, 7630.
[56] Abe, H.; Doi, Y.; Hori, Y.; Hagiwara, T. *Polymer*, **1997**, *25*, 185.
[57] Abe, H.; Doi, Y. *Int. J. Biol. Macromol.*, **1999**, *25*, 185.
[58] Tomasi, G.; Scandla, M.; Briese, B. H.; Jendrossek, D. *Macromolecules*, **1996**, *29*, 507.
[59] Abe, H.; Doi, Y.; Aoki, H.; Akehata, T. *Macromolecules*, **1998**, *31*, 1791.
[60] Kurokawa, K.; Yamashita, K.; Doi, Y.; Abe, H. *Polym. Degrad. Stab.*, **2006**, *91*, 1300.
[61] Kurokawa, K.; Yamashita, K.; Doi, Y.; Abe, H. *Biomacromolecules*, **2008**, *9*, 1071.
[62] Yamashita, K.; Aoyagi, Y.; Abe, H.; Doi, Y. *Biomacromolecules*, **2001**, *2*, 25.
[63] Yamashita, K.; Funato, T.; Suzuki, Y.; Teramachi, S.; Doi, Y. *Macromol. Biosci.*, **2003**, *3*, 694.
[64] Kikkawa, Y.; Yamashita, K.; Hiraishi, T.; Kanesato, M.; Doi, Y. *Biomacromolecules*, **2005**, *6*, 2084.
[65] Kikkawa, Y.; Fujita, M.; Hiraishi, T.; Yoshimoto, M.; Doi, Y. *Biomacromolecules*, **2004**, *5*, 1642.
[66] Murase, T.; Suzuki, Y.; Doi, Y.; Iwata, T. *Biomacromolecules*, **2002**, *3*, 312.
[67] Carrard, G.; Koivula, A.; Söderlund, H. S.; Béguin, P. *Proc. Natl. Acad. Sci. USA*, **2000**, *97*, 10342.
[68] Vaage-Kolstad, G.; Horn, S. J.; van Aalten, D. M. F.; Synstad, B.; Eijsink, V. G. H. *J. Biol. Chem.*, **2005**, *280*, 28492.

[69] Fujita, M.; Kobori, Y.; Aoki, Y.; Matsumoto, N.; Abe, H.; Doi, Y.; Hiraishi, T. *Langmuir*, **2005**, *21*, 11829.
[70] Behrends, A.; Klingbeil, B.; Jendrossek, D. *FEMS Microbiol. Lett.*, **1996**, *143*, 191.
[71] Nojiri, M.; Saito, T. *J. Bacteriol.*, **1997**, *179*, 6965.
[72] Hiraishi, T.; Ohura, T.; Ito, S.; Kasuya, K.; Doi, Y. *Biomacromolecules*, **2000**, *1*, 320.
[73] Kasuya, K.; Inoue, Y.; Tanaka, T.; Akehata, T.; Iwata, T.; Fukui, T.; Doi, Y. *Appl. Environ. Microbiol.*, **1997**, *63*, 4844.
[74] Shinomiya, M.; Iwata, T.; Kasuya, K.; Doi, Y. *FEMS Microbiol. Lett.*, **1997**, *154*, 89.
[75] Shinomiya, M.; Iwata, T.; Doi, Y. *Int. J. Biol. Macromol.*, **1998**, *22*, 129.
[76] Kasuya, K.; Ohura, T.; Masuda, K.; Doi, Y. *Int. J. Biol. Macromol.*, **1999**, *24*, 329.
[77] Ohura, T.; Kasuya, K.; Doi, Y. *Appl. Environ. Microbiol.*, **1999**, *65*, 189.
[78] Hiraishi, T.; Hirahara, Y.; Doi, Y.; Maeda, M.; Taguchi, S. *Appl. Environ. Microbiol.*, **2006**, *72*, 7331.
[79] Jendrossek, D.; Schirmer, A.; Handrick, R. In *1996 International Symposium on Bacterial Polyhydroxyalkanoates*; Eggink, G.; Steinbüchel, A.; Poirier, Y.; Witholt, B., Eds.; NRC Research Press, Ottawa, Canada, **1997**; pp. 89-101.
[80] Hisano, T.; Kasuya, K.; Tezuka, Y.; Ishii, N.; Kobayashi, T.; Shiraki, M.; Oroudjev, E.; Hansma, H.; Iwata, T.; Doi, Y.; Saito, T.; Miki, K. *J. Mol. Biol.*, **2006**, *356*, 993.
[81] Schulze, B.; Wubbolts, M. G. *Curr. Opin. Biotechnol.*, **1999**, *10*, 609.
[82] Ran, N.; Zhao, L.; Chen, Z.; Tao, J. *Green Chem.*, **2008**, *10*, 361.
[83] Gross, R. A.; Kaplan, D. L.; Swift, G. Eds. *Enzymes in Polymer Synthesis*, ACS Symposium Series 684; American Chemical Society, Washington, DC, **1998**.
[84] Gross, R. A.; Kumar, A.; Kalra, B. *Chem. Rev.*, **2001**, *101*, 2097.
[85] Kobayashi, S.; Uyama, H.; Kimura, S. *Chem. Rev.*, **2001**, *101*, 3793.
[86] Okumura, S.; Iwai, M.; Tominaga, Y. *Agric. Biol. Chem.*, **1984**, *48*, 2805.
[87] Matsumura, S.; Takahashi, J. *Makromol. Chem. Rapid. Commun.*, **1986**, *7*, 369.
[88] Uyama, H.; Kobayashi, S. *Chem. Lett.*, **1993**, 1149.
[89] Knai, D.; Gutman, A. L.; Kohn, D. H. *J. Polym. Sci. Part A, Polym. Chem.*, **1993**, *31*, 1221.
[90] Uyama, H.; Takeya, K.; Kobayashi, S. *Bull. Chem. Soc. Jpn.*, **1995**, *68*, 56.
[91] Uyama, H.; Kuwabara, M.; Tsujimoto, T.; Kobayashi, S. *Polym. J.*, **2002**, *34*, 970.
[92] Uyama, H.; Suda, S.; Kikuchi, H.; Kobayashi, S. *Chem. Lett.*, **1997**, 1109.
[93] Córdova, A.; Iversen, T.; Hult, K.; Martinelle, M. *Polymer*, **1998**, *39*, 6519.
[94] Kobayashi, S.; Takeya, K.; Suda, S.; Uyama, H. *Macromol. Chem. Phys.*, **1998**, *199*, 1729.
[95] Matsumura, S.; Harai, S.; Toshima, K. *Macromol. Rapid Commun.*, **2001**, *22*, 215.
[96] Soeda, Y.; Toshima, K.; Matsumura, S. *Macromol. Biosci.*, **2005**, *5*, 277.
[97] Kobayashi, S. *Macromol. Symp.*, **2006**, *240*, 178.
[98] van der Mee, L.; Helmich, F.; de Bruijn, R.; Vekemans, J. A. J. M.; Palmans, A. R. A.; Meijer, E. W. *Macromolecules*, **2006**, *39*, 5021.
[99] Nobes, G. A. R.; Kazlauskas, R. J.; Marchessault, R. H. *Macromolecules*, **1996**, *29*, 4829.
[100] Xie, W.; Li, J.; Chen, D.; Wang, P. G. *Macromolecules*, **1997**, *30*, 6997.
[101] Matsumura, S.; Suzuki, Y.; Tsukada, K.; Toshima, K.; Doi, Y.; Kasuya, K. *Macromolecules*, **1998**, *31*, 6444.
[102] Matsumura, S.; Beppu, H.; Nakamura, K.; Osanai, S.; Toshima, K. *Chem. Lett.*, **1996**, 795.
[103] Küllmer, K.; Kikuchi, H.; Uyama, H.; Kobayashi, *S. Macromol. Rapid Commun.*, **1998**, *19*, 127.
[104] Kikuchi, H.; Uyama, H.; Kobayashi, S. *Polym. J.*, **2002**, *34*, 835.
[105] Runge, M.; O'Hagan, D.; Haufe, G. *J. Polym. Sci. A. Polym. Chem.*, **2000**, *38*, 2004.
[106] Svirkin, Y. Y.; Xu, J.; Gross, R. A.; Kaplan, D. L.; Swift, G. *Macromolecules*, **1996**, *29*, 4591.
[107] Peeters, J. W.; Van Leeuwen, O.; Palmans, A. R. A.; Meijer, E. W. *Macromolecules*, **2005**, *38*, 5587.
[108] Al-Azemi, T. F.; Kondaveti, L.; Bisht, K. S. *Macromolecules*, **2002**, *35*, 3380.
[109] van Buijtenen, J.; van As, B. A. C.; Verbruggen, M.; Roumen, L.; Vekemans, J. A. J. M.; Pieterse, K.; Hilbers, P. A. J.; Hulshof, L. A.; Palmans, A. R. A.; Meijer, E. W. *J. Am. Chem. Soc.*, **2007**, *129*, 7393.

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